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In Vitro Efficacy of Brincidofovir against Variola Virus

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Brincidofovir (CMX001), a lipid conjugate of the acyclic nucleotide phosphonate cidofovir, is under development for smallpox treatment using "the Animal Rule," established by the FDA in 2002. Brincidofovir reduces mortality caused by orthopoxvirus infection in animal models. Compared to cidofovir, brincidofovir has increased potency, is administered orally, and shows no evidence of nephrotoxicity. Here we report that the brincidofovir half-maximal effective concentration (EC_{50}) against five variola virus strains *in vitro* averaged 0.11 μM and that brincidofovir was therefore nearly 100-fold more potent than cidofovir.

Although smallpox was declared eradicated by the World Health Organization in 1980, the etiologic agent (variola virus [VARV]) remains a category A select agent (subject to select agent regulations [42CFR, part 73]) or a "Highest Priority" biological threat due to its high mortality rate and ease of transmission (1). Vaccination using a closely related live orthopoxvirus (vaccinia virus) prevents smallpox but is associated with potentially severe complications and therefore is not recommended for routine use in the absence of an immediate threat of a VARV release or smallpox outbreak. The vaccine is also contraindicated in immunocompromised individuals, including the very young or old, pregnant women, and those receiving immunosuppressive therapies (2). In the event of a VARV release, there would be a need for antiviral drugs to treat individuals exposed to or infected with smallpox.

Brincidofovir (BCV, CMX001, hexadecyloxypropyl-cidofovir [HDP-CDV]), a lipid-conjugated acyclic nucleotide phosphonate, has broad-spectrum *in vitro* activity against double-stranded DNA viruses, including herpesviruses, adenoviruses, and poxviruses (3). BCV has completed two phase 2 clinical trials for the prevention of clinically significant cytomegalovirus infection in hematopoietic stem cell transplant patients and is currently in phase 3 trials. Simultaneously, BCV has been in development for the treatment of smallpox under the Animal Rule, which states that when developing medical countermeasures for threat agents where human challenge studies are not ethical or feasible (e.g., VARV), FDA may grant approval based on animal model studies which demonstrate that the drug is reasonably likely to have clinical benefit in humans (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078923.pdf) (3, 4).

Upon entering a cell, the lipid moiety of BCV is cleaved to release free cidofovir, which is then phosphorylated to cidofovir diphosphate (CDV-PP). CDV-PP inhibits viral DNA polymerase by serving as an alternate substrate, resulting in the inhibition of viral DNA synthesis (5–7). Among the orthopoxviruses, BCV has proven activity in animal models against vaccinia virus, rabbitpox virus (a subspecies of vaccinia), and ectromelia virus and *in vitro* against monkeypox virus (3). BCV has also been shown to be active against VARV *in vitro* (8); however, due to regulations restricting its use, these data are limited.

Since BCV ultimately acts via inhibition of the viral DNA polymerase, bioinformatic analysis of 47 sequenced strains of variola virus (9) was used to identify differences within the E9L viral DNA polymerase protein. Five strains of variola virus were selected for testing to represent the five distinct E9L genotypes (see Fig. S1 in the supplemental material). The origins of the five different strains (VARV_SOM77_ali, VARV_ BSH74_sol, VARV_BRZ66_39, VARV_JAP51_stwl, and VARV_UNK52_but) have been previously published (9). Viruses were propagated on African green monkey kidney cells (BSC-40), and crude preparations of virus were harvested at 48 or 72 h postinfection as previously described (10).

The cell-clearing plaque assay to determine the half-maximal effective concentration (EC_{50}) was performed as previously described (11) with minor modifications. BSC-40 cells were seeded in 96-well plates and grown to confluence. Cells were infected with each virus strain at a multiplicity of infection of 0.1. Virus was allowed to infect for 1 h at 35.5°C and 6% CO_2. The inoculum was removed, and medium (RPMI medium–2% fetal bovine serum [FBS]) alone or containing various concentrations of compound (BCV at 2-fold dilutions of 10 μM to 0.005 μM or cidofovir at 2-fold dilutions of 100 μM to 0.5 μM) was added to the infected wells. Each treatment was performed in triplicate. Uninfected cells were also incubated with medium containing each compound to determine cellular toxicity. The plates were incubated for 3 days, and the cells were then fixed with 2× crystal violet stain (10% EtOH, 60% formalin, 30% double-distilled water [ddH_2O], and 0.26% crystal violet). The stained viable cells were quantified by measuring the optical density at 570 nm. Dose-response curves were generated by combining data from the triplicate wells for each virus strain (see Fig. S2 and S3 in the supplemental material). The effective concentration of compound that protected the monolayers at the 50% level (EC_{50}) was calculated from the absorbance values by using GraphPad Prism software (version 5).

BCV was active against each of the five variola virus strains tested, with EC_{50}s ranging from 0.05 μM to 0.21 μM (Table 1).

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TABLE 1 EC_{50}s for different variola virus strains

Compound	EC_{50} value (μM) for strain:				
	BSH74	SOM77	JPN51	UNK52	BRZ66
BCV	0.21	0.077	0.11	0.05	0.11
Cidofovir	6.07	1.37	10.81	7.08	28.45

Cidofovir was also active against each of the five strains but was 97-fold less potent on average (range, 18-fold to 259-fold). There are no apparent commonalities in linear alignments of the E9L polymerase protein sequences (see Fig. S1 in the supplemental material) that account for differences in EC_{50} between strains of variola virus; however, it remains possible that amino acid differences produce structural changes which alter the ability of the different polymerases to incorporate CDV-PP into the elongating VARV DNA. The cytotoxic concentration (CC_{50}) of BCV for uninfected cells was extrapolated to be approximately 15 μM, yielding an average selectivity value of 135-fold.

These results are consistent with the previously reported BCV EC_{50} of 0.1 μM which was determined using variola virus strain BSH (8). The BCV EC_{50}s reported for other orthopoxviruses are in a similar range (0.07 to 0.8 μM), as summarized previously (3). These results show that BCV is an effective agent against multiple variola virus strains *in vitro*. Previous studies have demonstrated *in vivo* antiviral activity of BCV in numerous animal models of orthopoxvirus infection, including ectromelia virus in mice and rabbitpox virus in rabbits (12, 13). Therefore, the current results support further development of BCV for the treatment of smallpox.

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